

**AMENDMENTS TO THE SPECIFICATION:**

**Please amend the specification as follows:**

**Replace the paragraph beginning at page 3, lines 10-16 with the following amended paragraph:**

In another aspect of this embodiment, the known protease scaffold can include the amino acid sequence of trypsin, chymotrypsin, subtilisin, thrombin, plasmin, Factor Xa, urokinase type plasminogen activator (uPA), tissue plasminogen activator (tPA), membrane type serine protease-1 (MTSP-1), granzyme A, granzyme B, granzyme M, elastase, chymase, papain, neutrophil elastase, plasma kallikrein, ~~urokinase type plasminogen activator~~, complement factor serine proteases, ADAMTS13, neural endopeptidase/neprilysin, furin, or cruzain.

**Replace the paragraph beginning at page 3, lines 29-30 with the following amended paragraph:**

In another aspect of this embodiment, the substrate sequence in a library is 4, 5, 6, 7, 8, 9, 10, 11, [[11,]] 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acids long.

**Replace Table 1 with the following table:**

Target	Indication	Molecule class
IL-5/IL-5R	Asthma	Cytokine
IL-1/IL-1R	Asthma, inflammation, Rheumatic disorders	Cytokine
IL-13/IL-13R	Asthma	Cytokine
IL-12/IL-12R	Immunological disorders	Cytokine
IL-4/IL-4R	Asthma	Cytokine
TNF/TNFR	Asthma, Crohn's disease, HIV infection, inflammation, psoriasis, rheumatoid arthritis	Cytokine
CCR5/CXCR4	HIV infection	GPCR
gp120/gp41	HIV infection	Fusion protein
CD4	HIV infection	Receptor
Hemagglutinin- Hemagglutinin	Influenza infection	Fusion protein
RSV fusion protein	RSV infection	Fusion protein
B7/CD28	Graft-v.-host disorder, rheumatoid arthritis, transplant rejection, diabetes mellitus	Receptor
IgE/IgER	Graft-v.-host disorder, transplant rejection	Receptor
CD2,CD3,CD4,CD40	Graft-v.-host disorder, transplant rejection, psoriasis,	Receptor
IL-2/IL-2R	Autoimmune disorders, graft-v.-host disorder, rheumatoid arthritis	Cytokine
VEGF,FGF,EGF,TGF	Cancer	Cytokine
Her2/neu	Cancer	Receptor
CCR1	Multiple sclerosis	GPCR
CXCR3	Multiple sclerosis, rheumatoid arthritis	GPCR
CCR2	Atherosclerosis, rheumatoid arthritis	GPCR
Src	Cancer, osteoporosis	Kinase
Akt	Cancer	Kinase
Bcl-2	Cancer	Protein-protein
BCR-Abl	Cancer	Kinase
GSK-3	Diabetes	Kinase
cdk-2/cdk-4	Cancer	Kinase

**Replace the paragraph beginning at page 17, line 27 through page 18, line 7 with the following amended paragraph:**

In another embodiment of the invention, scaffold proteases are chosen using the following requirements: 1) The protease is a human or mammalian protease of known sequence; 2) the protease can be manipulated through current molecular biology techniques; 3) the protease can be expressed heterologously at relatively high levels in a suitable host; and 4) the protease can be purified to a chemically competent form at levels sufficient for screening. In other embodiments of the invention, the scaffold protease to be mutated cleaves a protein that is found extracellularly. This extracellular protein is, for example, a receptor, a

signaling protein, or a cytokine. The residues that, upon mutation, affect the activity and specificity of two families of scaffold proteases are described here. Preferably, ~~there is~~ three dimensional structural information for the protease is available. Also, it is preferred that there be knowledge of the initial substrate specificity of the protease. It is also preferable that the protease be active and stable *in vitro* and that knowledge of macromolecular modulators of the protease are available. Also, proteases are preferred which cleave targets that are relevant to affecting pathology, *e.g.* inactivating protein effectors of pathology.

**Replace the paragraph beginning at page 19, line 27 through page 20, line 6 with the following amended paragraph:**

To make a variant protease with an altered substrate recognition profile, the amino acids in the three-dimensional structure that contribute to the substrate selectivity (specificity determinants) are targeted for mutagenesis. For the serine proteases, numerous structures of family members have defined the surface residues that contribute to extended substrate specificity (Wang *et al.*, Biochemistry 2001 Aug 28;40(34):10038-46; Hopfner *et al.*, Structure Fold Des. 1999 Aug 15;7(8):989-96; Friedrich *et al.* J Biol Chem. 2002 Jan 18;277(3):2160-8; Waugh *et al.*, Nat Struct Biol. 2000 Sep;7(9):762-5). Structural determinants for various proteases are listed in Table 3, along with a listing of the amino acid in a subset of family members determined to be of known, extended specificity. For serine proteases, the following amino acids in the primary sequence are determinants of specificity: 195, 102, 57 (the catalytic triad); 189, ~~190, 191~~, 190, 191, 192, and 226 (P1); 57, the loop between 58 and 64, and 99 (P2); 192, 217, 218 (P3), the loop between Cys168 and Cys180, 215 and 97 to 100 (P4).

**Replace the paragraphs beginning at page 22, lines 9-26, with the following amended paragraphs:**

For the P3 and P4 subsites, mutations at Tyr174, Arg192 and Asn218 did not significantly affect the specificity (~~See Table 4~~ Table 5, below). Y174A increases the activity towards Leu at P4, but the rest of the amino acids continue to be poorly selected. R192A and N218A both broaden the specificity at P3. Instead of a strong preference for glutamic acid, Ala, Ser, Glu and Gln are similarly preferred in the mutant. The overall activity (kcat/Km) of the mutant is less than 10% below the wild type activity toward an ideal wild-type substrate, N-acetyl-Ile-Glu-Pro-Asp-AMC (7-amino-4-methylcoumarin) (Ac-IEPD-AMC) (SEQ ID NO: 6).

A much more dramatic effect is observed at the P2 subsite (~~See Table 4~~ Table 5, below). In wild type granzyme B, the preference is broad with a slight preference for Pro

residues. 199A narrows the P2 specificity to Phe and Tyr residues. Phe is now preferred nearly 5 times over the average activity of other amino acids at this position. Within the chymotrypsin family of serine proteases, more than a dozen proteases have a small residue at this structural site, either an asparagine, serine, threonine, alanine or glycine. From this group, two proteases have been profiled using combinatorial substrate libraries, (plasma kallikrein and plasmin), and both show strong preferences towards Phe and Tyr. These two results suggest that any serine protease that is mutated to an Asn, Ser, Thr, Gly or Ala at position 99 will show the same hydrophobic specificity found in plasma kallikrein, plasmin and the 199A granzyme B mutant.

**Replace the paragraph beginning at page 25, lines 19-28, with the following amended paragraph:**

The protease may be expressed in an active or inactive, zymogen form. The protease may be in a heterologously expressing system such as ~~*E. coli*~~ *E. coli*, *Pichia pastoris*, *S. cerevisiae*, or a baculovirus expression system. The protein can either be expressed in an intracellular environment or excreted into the media. The protease can also be expressed in an *in vitro* expression system. To purify the variant protease, column chromatography can be used. The protease may contain an C-terminal 6-His tag for purification on a Nickel column. Depending on the pI of the protease, a cation or anion exchange column may be appropriate. The protease can be stored in a low pH buffer that minimizes its catalytic activity so that it will not degrade itself. Purification can also be accomplished through immunoabsorption, gel filtration, or any other purification method commonly used in the art.

**Replace the paragraph beginning at page 31, lines 15-27, with the following amended paragraph:**

In another preferred embodiment, these methods are used to select for an enzyme that specifically cleaves a target sequence, and preferably for an enzymatically active protease. The method includes: (a) a random peptide library containing an internally quenched fluorophore, where the fluorophore is *e.g.* o-aminobenzoyl and the quencher is *e.g.* 3-nitrotyrosine; (b) a peptide substrate sequence corresponding to the sequence targeted for cleavage, which also contains an internally quenched fluorophore where the fluorophore is *e.g.* Cy3B and the quencher is *e.g.* Cy5Q; (c) mixing the random peptide library and peptide substrate sequence at a 1:1 ratio; (d) exposing the mixture to the mutant protease and then quantitating the ratio of Cy3B fluorescence to o-aminobenzoyl fluorescence. If a protease is selective for the target peptide, it will cleave only the target peptide and not the random library, and thus there will be a high ratio of Cy3B fluorescence to o-aminobenzoyl

fluorescence. (Meldal and Breddam, Anal. Biochem. (1991) 195: 141-147; Gron, *et al.* Biochemistry (1992) 31: 6011-6018)

**Replace the paragraph beginning at page 38, lines 25-31, with the following amended paragraph:**

In one aspect, the invention provides a method of treating or preventing an apoptosis-associated disorder in a subject in need thereof by administering to the subject a therapeutically effective amount of a protease-inhibitor so apoptosis is inhibited. The subject can be *e.g.*, any mammal, *e.g.*, a human, a primate (~~*e.g.*, a human~~), mouse, rat, dog, cat, cow, horse, or pig. The term "therapeutically effective" means that the amount of protease-inhibitor, for example, which is used, is of sufficient quantity to ameliorate the apoptosis-associated disorder.

**Replace the paragraph beginning at page 39, line 32 through page 40, line 3 with the following amended paragraph:**

Also included in the invention are methods of inducing apoptosis. In one aspect apoptosis is induced in subject in need thereof by administering a protease in an amount sufficient to induce apoptosis. The subject can be *e.g.*, any mammal, a primate (~~*e.g.*, a human~~), mouse, rat, dog, cat, cow, horse, or pig. In various aspects the subject is susceptible to cancer or an autoimmune disorder.

**Replace the paragraph beginning at page 41, line 25 through page 42, line 3 with the following amended paragraph:**

The DNA containing the variant granzyme B proteases was transformed into ~~*Pichia pastoris*~~ *Pichia pastoris* X33 cells by the published protocol (Invitrogen) and the positive transformants were selected with Zeocin. The colony was transferred to a 1 L liquid culture and grown to a cell density of greater than OD<sub>600</sub> = 1.0. Protein expression was induced by the addition of 0.5% methanol and held constant over 72 hours. To purify the variant protease, the culture was centrifuged and the supernatant collected. Gravity based loading flowed the supernatant over a SP-Sepharose Fast Flow cation exchange column. The column was washed with 50 mM MES, pH 6.0, 100 mM NaCl, and more stringently with 50 mM MES, pH 6.0, 250 mM NaCl. The protein was eluted with 50 mM MES, pH 6.0, 1 M NaCl and the column washed with 50 mM MES, pH 6.0, 2M NaCl and 0.5 M NaOH. The resulting protease was <90% pure. The final protease was exchanged and concentrated into 50mM MES, pH 6.0, 100 mM NaCl for storage at 4 °C.

**Replace the paragraph beginning at page 44, lines 15-3, with the following amended paragraph:**

This method was used to screen I99A granzyme B. I99A granzyme B was profiled in a positional scanning combinatorial substrate library to determine the effect of the mutation. The library was prepared as described above and aliquoted into 96-well plates to a final concentration of 250  $\mu$ M. The variant protease was diluted in granzyme activity buffer (50 mM Na HEPES, pH 8.0, 100 mM NaCl, 0.01% Tween-20) to concentrations between 50 nM and 1  $\mu$ M. Initial activity against Ac-IEPD-AMC was used to adjust the variant protease concentration to one approximately equal to 50 nM wild type rat granzyme B. Enzymatic activity in the P1-Asp library was assayed for one hour at 30 °C on the Spectra-Max Delta fluorimeter. Excitation and emission were measured at 380 nm and 460 nm, respectively. The profiles of the granzyme B variants were compared to the wild type profile and the differences determined. For the I99A mutant, for example, the specificity at the P2 amino acid was markedly changed from the wild type. The former broad preference with a slight preference for proline is replaced with a strong preference for hydrophobic residues such as Phe and Tyr. The selectivity of the variant protease was also changed. The wild type was promiscuous at the P2 subsite, hydrolyzing substrates that contain any amino acid at that site. The I99A protease is much more selective. A Phe at the P2 site is preferred to a much higher degree than any other amino acid (*See Table 5, above*).

**Replace the paragraph beginning at page 45, lines 10-12, with the following amended paragraph:**

<sup>125</sup>I-TNF (40,000 cpm) is incubated with varying concentrations of protease and then samples are boiled in SDS-PAGE sample buffer and examined on a 12% polyacrylamide gel. Gels are dried and exposed to x-ray ~~film(Kodak)~~ film (Kodak) at -70 °C.

**Replace the paragraph beginning at page 49, lines 17-19, with the following amended paragraph:**

<sup>125</sup>I-TNF (40,000 cpm) is incubated with varying concentrations of protease, samples are boiled in SDS-PAGE sample buffer and examined on a 12% polyacrylamide gel. Gels are dried and exposed to x-ray ~~film(Kodak)~~ film (Kodak) at -70 °C.

**Replace the paragraph beginning at page 52, lines 1-5, with the following amended paragraph:**

Figure 1 shows the sequence of caspase-3, a protein implicated in the apoptosis pathway of many cell types. Wild-type granzyme B cleaves ~~easpase-3~~ caspase-3 between the aspartate and serine at residues 175 and 176 respectively. Mutations at positions 99 and 218

Applicant : Nguyen *et al.*  
Serial No. : 10/677,977  
Filed : October 2, 2003

Attorney's Docket No.: 0119357-00007/4905  
**Amendment and Response**

of granzyme B, change the specificity of this protease to cleavage between the aspartate and alanine of residues 263 and 264 respectively.